GEORGIA INSTITUTE OF TECHNOLOGY · · OFFICE OF CONTRACT ADMINISTRATION

[x] ORIGINAL

[ ] REVISION NO. ____

PROJECT ADMINISTRATION DATA SHEET

Project No. G-32-C03 DATE: 6/24/81

Project Director: ~~Dr. W. F. Balo~~ María de los Angeles Contreras School/Lab XXX Biology

Sponsor: DHEW/PHS/NIH-National Cancer Institute, Bethesda, MD 20014

Type Agreement: Grant No. 5 RO1CA25958-03

Award Period: From 7/1/81 To ~~6/30/82~~ 12/31/82 (Performance) 9/30/82 (Reports)

Sponsor Amount: $121,174 Contracted through: XXXX ~~GTRI~~/GIT

Cost Sharing: $6,027 (G-32-335)

Title: Organ Specific Tumor Localizing Antibody

ADMINISTRATIVE DATA OCA CONTACT Faith G. Costello

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Reports: See Deliverable Schedule Security Classification: N/A

Defense Priority Rating: N/A

RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements

Travel: Foreign travel must have prior approval - Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with GIT usually; however, we are accountable for all equipment purchased.

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GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION SHEET

Date 1/12/83

Project Title: Organ Specific Tumor Localizing ~~Centibody~~ Antibody

Project No: G-32-C03

Project Director: Maria de los Angeles Contraras

Sponsor: DHHS/PHS/NIH - National Cancer Institute

Effective Termination Date: 2/31/82

Clearance of Accounting Charges: 2/31/82 (Performance)
3/31/83 (Reports)

Grant/Contract Closeout Actions Remaining:

- ☐ Final Invoice and Closing Documents
- ☒ Final Fiscal Report
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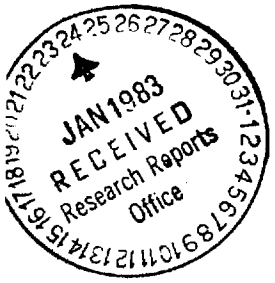

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FORM OCA 10:781

Terminal Progress Report

Grant ..... #5 R01 CA 2595803

Title ..... Organ Specific Tumor Localizing Antibody

Principal Investigator .. William F. Bale

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Final Report

Grant #5 R01 CA 2595803

Funded at the School of Applied Biology, Georgia Institute of Technology from July 1, 1979 to June 30, 1982.

At the end of May 1982, Dr. Bale became seriously ill, and a six-month extension to this grant was requested by the Director of the School of Applied Biology, Dr. Thomas G. Tornabene, on June 18, 1982.

On June 28, 1982, Dr. Bale passed away. On July 18, 1982, Dr. Thomas G. Tornabene requested that I, M. Angeles Contreras, be approved as Acting Project Director in completing the remaining testing and reporting for this grant for a period of six months at no cost. In your letter dated July 29, 1982, you approved my appointment as a Principal Investigator of this grant through December 31, 1982.

Principal Investigator ..... William F. Bale
Interim Principal Investigator ..... M. Angeles Contreras

Title ..... Organ Specific Tumor Localizing Antibody

Part 1. Production of an immunological probe.

This research program was based on the premise that tumors originating in organs not essential to life and well-being sometimes express cell surface antigens unique to those organs, and that antibodies to these antigens may be useful in the treatment of such tumors.

Induction of DMBA tumors in imbred F-344 rats.

Tumors were induced in imbred F-344 rats by stomach administration of DMBA. It took 2-8 months for the rats to develop the tumors. A mammary tumor was surgically removed from each rat as tumors developed, and a tumor line from each rat was established by subcutaneous implant in the same strain of rats. The first tumor line is now in the 37th generation. A total of 8 tumor lines were established. One rat developed an ovarian tumor. One spontaneous tumor arose in an aging F-344 rat.

Tumor antigen preparation. From each of the first generation tumor lines, tumor antigen was prepared using either tumor cell membranes or by explosive decompression of tumor cells (Parr nitrogen bomb) followed by differential centrifugation in sucrose gradient.

Antibody production. Xenogenic antibodies were produced by immunizing rabbits with tumor cell membranes. For immunization this antigen was combined with complete Freund adjuvant.

Antibody titration. Individual serum from rabbits immunized with tumor cell preparation were carried out using a second antibody. This technique will be described under pancreas antibody titration.

Antibody purification. In these studies, immune sera were absorbed and eluted onto polymerized normal rat tissues (excluding mammary tissue) in 2X concentrated normal rat serum. The best purification that we were able to obtain consisted in first absorbing and eluting the polymerized normal tissues 3X at pH 3.2. Then we used this 3X absorbed polymer for antisera absorption. From this absorbed antisera γ-globulin was prepared. Then this γ-globulin was absorbed and eluted onto 3X eluted polymerized tumor membranes. The eluate was concentrated by negative pressure, then labeled with ^{125}I. This ^{125}I-labeled *in vitro* purified preparation was *in vivo* screened.

When *in vitro* tested, this labeled preparation showed a slightly decreased binding to normal rat kidney, liver and spleen, but no increase in tumor binding was obtained.

When *in vivo* tested, this ^{125}I-labeled preparation showed moderate preferential localization after it was intravenously injected to tumor-bearing rats in all DMBA tumor lines tested. In some experiments an attempt was made

to reisolate the antibody which had localized in these tumors. We were able to elute the antibody, but the levels of radioactivity were low and no attempt to reinject into tumor-bearing rats was made.

Discussion and conclusions. Our experimental results indicate that DMBA induced mammary tumors in rats shared common antigens on the surface of tumor cells that can lead to ^{125}I-labeled antibody localization in tumors after intravenous injection. We have some, although not conclusive, evidence that this antibody is able to localize in normal mammary tissue. We do not have data that excludes effects of viruses or other microorganisms that may be present and pass by tumor transplant from rat to rat.

A second related conclusion is that although our data demonstrates tumor localizing antibody, the production of this specific antibody following our methods of immunization is a very small fraction of the total antibody response.

It also seems quite clear that the procedures for specific antibody production and purification so far described here are too inefficient for any extensive studies of tumor localizing antibodies.

Pancreas membranes antigen. Pancreas was disected out from rats under halothene anesthesia. Several enzyme inhibitors were used to prevent outolysis. After homogenization and several washes and centrifugations, the pancreas membranes were resuspended in 0.85% saline. Xenogenic antibodies were produced by immunizing rabbits with pancreas membranes in complete Freund adjuvant.

Antibody titration. We developed a method for the titration of unlabeled rabbit antisera or sera fractions to rat organ or tumors. This method uses ^{125}I-labeled purified goat antibody to rabbit γ-globulin. A fixed amount of antigen was incubated with a fixed dilution of immune serum. The washed portions of antigen now binding to antibody were incubated with ^{125}I-labeled goat antibody containing progressible amounts of the same but unlabeled goat antibody to rabbit γ-globulin. This method was used for titrations of immune sera to rat mammary tumors before purification and ^{125}I-labeling.

Studies with antibodies to rat pancreas. Experiments were conducted to isolate pancreas-specific antibody by different elution techniques, either before and after ^{125}I-labeling. These techniques were not successful. Most of the ^{125}I-labeled antibody bound to polymerized pancreas membranes, on elution turned out to be largely hydrolysed. The few trypsin inhibitors tested seemed relatively ineffective in preventing this hydrolysis.

Discussion and conclusions. Our experimental results indicate that a very small fraction of the pancreas-specific γ-globulin binds to some extent to pancreas tissue after intravenous injection. We do not know whether antibody binding takes place in the cell surface antigen. Our data indicates that this binding is extremely slow and perhaps the eluted antibody may be complex with soluble antigens and is catabolized very rapidly from the rat circulation. This may be one explanation for the high values of ^{125}I found in the spleen.

Part II. Studies related to the possible therapeutic use of specific antibody and corresponding antigen.

Rate of penetration of blood γ-globulin into the extravascular-extracellular space of tumors. In vivo screened ^{125}I-labeled normal rabbit γ-globulin was injected IV into rats bearing subcutaneous kidney and liver tumor implants. A second injection of ^{131}I-labeled normal rabbit γ-globulin was given IV, 24 or 48 hours later. Ten minutes after the last injection rats were sacrificed. The rate at which ^{125}I and ^{131}I accumulated in tumors was in all cases higher in tumor than in normal tissue.

These results give some evidence that this physiological property of tumors of γ-globulin sequestration in the extravascular-extracellular space of tumors could be of some use for possible application in boron neutron capture therapy, if the circulating boron covalented attached to γ-globulin could be rapidly removed from the blood stream at the time in which boron concentration in the tumor is high. Two possibilities were sought. The first possibility was to develop an immunological technique. The second possibility was to develop a method for a rapid blood exchange in the rat.

Immunological technique for removing labeled normal IGg from tumor-bearing rat circulation. ^{125}I-labeled rabbit IGg supplemented with normal rabbit serum was injected IV into rats bearing either subcutaneous, kidney or liver tumor implant. Then 24 hours later, a second injection of goat anti-rabbit γ-globulin containing the predeterminated units of goat antibody needed to precipitate all rabbit γ-globulin circulating in the rat blood was given. The rats were sacrificed 30 min. after the last injection was administered.

Discussion and conclusions. Our results indicate that the possibilities of this technique are of important significance for removing almost any unwanted circulating chemotherapeutic agents covalently attached to γ-globulin at the time when tumor concentrations are high. Some side effects observed in rats follow: on kidney implanted tumors, there was a trasient slight hemorrhage in the small intestine; on liver implanted tumors this hemorrhage was observed in colon and cecum. This hemorrhage was not present 48 hours after the second injection.

The second possibility is being investigated at the present time by Miss. Denise J. Noonan, a Nuclear Engineer Ph.D. cancicate who was working under Dr. Bale's supervision in the dosimetry of boron neutron capture therapy.

Our studies with organ tumor specific antibodies were not successful for any attempted therapeutic use. Therefore we did not pursue these studies further. Nontheless, both pancreas and mammary tissue antibodies showed a fair amount of avidity for these pancreas, mammary tumors and normal mammary tissues. Labeled antibodies remained bound to these tissues 6-8 days after being intravenously injected in the rat.

With the development of more sophisticated methods it might be possible to continue this line of study.

Organ Specific Tumor Localizing Antibody

1. (a) M. Angeles Contreras, William F. Bale, and Irving L. Spar. Iodine Monochloride (ICl) Iodination Techniques. Methods in Enzymology. Vol. 92 (In Press).

2. No changes.

3. Progress report.

A. Objectives:

The overall purpose of this project was to demonstrate the truth of the assumption that malignant tumors, arising in organs not vital to life and well-being, express organ specific antigens that can lead to highly preferential localization in such tumors after injected radioactive antibodies of the proper specificity.

The principle goals for this year were to continue our efforts on the production of antibodies localizing in rat mammary tumors.

B. Studies conducted during the current year:

Here we will first describe, in sequence, a series of experimental procedures that lead to labeled antibody preparations that provide evidence of preferential labeled antibody localization in rat mammary tumors. We extended our studies on antibody purification by absorbing and eluting the polymerized normal rat tissues (excluding mammary tissue) at pH 3.2. Protein determination was followed on each eluate by the Lowry method, until the optical density in eluate was equal to the blank. A total of 3 absorptions and elutions were required. IGg was prepared from 5.0 ml of a rabbit immunized with first generation carcinogen induced Line 1 mammary tumor membrane preparation. This IGg was absorbed and eluted onto the 3X absorbed and eluted polymerized normal rat tissues. The eluted IGg was then absorbed and eluted onto polymerized tumor membranes, which had been previously absorbed and eluted 3X at pH 3.2. Eluate was concentrated by negative pressure dialyses. Protein content was assayed and labeled with ^{125}I. The total protein recovered was 1.6 mg on 0.5 ml. For labeling we used a modification of the ICl microiodination method (in press). Briefly, this method is as described for the microiodination, except in these iodinations we used the ^{125}I con-V shipping vial to carry out the iodination. 2.62 mCi of ^{125}I were attached to the antibody. This labeled antibody was *in vivo* purified in 2 male F-344 rats for 24 hours. Rats were on non-radioactive KI drinking water 24 hours before 1.2 mCi of labeled antibody was IV injected. Rats were bled to death by heart puncture. Serum was collected and pooled. A total of 105 µCi of labeled antibody was recovered in the sera. Ninety-three µCi of this *in vivo* screened antibody was absorbed and eluted onto 3X eluted polymerized tumor antigen. This eluted labeled antibody contained 1.8 µCi of ^{125}I. The per cent elution was 64.2 and the overall yield was 1.93%. *In vitro* testing showed that this labeled antibody had slightly decreased the uptake in the 2 tumor lines tested, and a marked decrease in normal rat and rabbit kidney. When *in vivo* testing in F-344 mammary tumor bearing rats, tumor localization did not increase but normal mammary gland tissue showed localization close to the values found in tumor. These values will be given in the next section. As Dr. Bale pointed out this purification

system thus far described may be attributed to antigens that are not present on the tumor cell surface or are otherwise masked in vivo and thus this antibody does not preferentially bind in tumor after intravenous injection. Another possibility is that organ specific antibodies react with antigen(s) or with circulating blood components and are catabolized before they reach the tumor; these possibilities are indicated by the high levels of radioactivity found in the spleen and liver.

Reisolation of labeled antibody bound to tumor. A similar antibody purification and ^{125}I-labeling was done. This labeled antibody was sephadex G-200 filter. IGg fractions pooled. To this ^{125}I-labeled antibody, ^{131}I in vivo screened normal rat IGg was added. This mixture was given IV to 4 F-344 bearing subcutaneous mammary tumors. All rats were sacrificed by guillotine. One rat was killed 24 hours after injection, 2 were killed at 65 hours and one at 164 hours. ^{125}I and ^{131}I content of blood, tumor and other rat tissues was measured, then calculated as a per cent of the injected dose in tissue equal to 1% of the rat weight. ^{125}I values for representative tissues follows: for 24 hours, blood 0.75, tumor 0.96 and 0.99, mammary gland 0.90, spleen 3.41, liver 1.34, kidney 1.31, skin 1.08 and muscle 1.03. Tumor weight was 1.16 grs. The average for the 2 rats killed at 64 hours follows: blood 0.69, tumor 0.80, mammary gland 0.98, spleen 3.86, liver 1.14, kidney 1.30, skin 0.91 and muscle 0.91. Average tumor weight was 1.82 gr. For the rat killed at 164 hours values were: blood 0.53, tumor 0.76, mammary gland 0.05, for ^{125}I, no ^{131}I was found, spleen 14.35, liver 1.14, kidney 1.61, skin 0.61, muscle 0.79, tumor weight was 10.56 gr. After the ^{125}I and ^{131}I content in tumors was counted, each tumor was homogenized in 0.85% NaCl, and then centrifuged. ^{125}I and ^{131}I contents in residues and supernates were measured and then calculated as per cent of the injected dose in tissue equal to 1% of the rat weight. ^{125}I found in residues of 24, 65 and 164 hours were 2.38, 2.71 and 1.35; in the same order amount of ^{125}I found in supernates were 0.61, 0.53 and 0.46. Although these values seem higher, they are still too low to be of any use for antigen isolation. The antibody preparations we were able to obtain do show moderate preferential localization, and to some extent some avidity for mammary tumor after 165 hours.

Accumulation of ^{125}I-labeled normal rabbit IGg in the extravascular-extracellular space in tumors and normal tissues. Dr. Bale was, for many years, interested in the extravascular-extracellular space in experimental rat tumors. (He directed two Ph.D. theses in this subject.) To further document these studies, we implanted a DMBA induced mammary tumor subcutaneously into the right flank of F-344 rats, and studied the accumulation of ^{125}I and ^{131}I-labeled normal rabbit IGg. Both ^{125}I and ^{131}I-labeled normal rabbit IGg were labeled by the ICl micro-iodination method. Both radioactive γ-globulins were in vivo screened for 24 hours. Rats were on non-radioactive KI drinking water 24 hours before 100,000 cpm/0.5 ml of ^{125}I-labeled γ-globulin injection was given IV. Twenty-four hours later 100,000 cpm/0.5 ml of ^{131}-labeled γ-globulin was IV injected. Rats were killed by guillotine 10 minutes after the last injection. Distribution of ^{125}I and ^{131}I in blood and tissue is given as a per cent of the injected ^{125}I and ^{131}I doses found in blood or tissues equal to 1% of the rat weight. ^{125}I tumor to blood ratio were higher by a factor of 15 in tumor, ratios for ^{125}I organ to to blood were in the order of 1 to 2 for spleen, kidney, liver and lungs. Additional experiments were carried out in DMBA induced tumors implanted surgically into the F-344 rats' liver. In these studies rats were given ^{125}I-in vivo

screened labeled normal rabbit IGg, then 1 or 2 days later sacrificed by perfusion with 0.85% NaCl through the vena cava. Data are given as the ratio of ^{125}I found in tumor or liver/^{125}I found in blood in per cent of the injected dose equal to 1% of the rat weight. The amount of ^{125}I found in tumor was 0.49 and for normal liver 0.06 for rats sacrificed after 24 hours. For rats sacrificed after 48 hours the values for tumor were 0.35 and for normal liver 0.067. Additional experiments were carried out in surgically implanted tumors in the F-344 rat's kidney. Results were similar to those found for subcutaneous and liver tumors. These data, although limited, seem to indicate that tumors growing in the liver and in the kidney may sequester injected γ-globulin in the extravascular-extracellular space in a way closely resembling subcutaneous implanted tumors. Values for tumor, liver and spleen were lower than those obtained with tumor specific antibody. It is possible that our preparations of organ and tumor specific antibodies reacted with antigen circulating in the blood stream before reaching the tumor. Our results indicate that this property of tumors may be of use in overcoming one of the major difficulties associated with therapy of human cancer tumors inside the abdomen and thorax metastatic growth.

Immunological technique for removing labeled normal rabbit IGg from tumor-bearing rat circulation. We spent most of this year on this technique. This technique originally developed by Spar and co-workers in Dr. Bale's laboratory has been used to remove ^{131}I-labeled rabbit antibody to rat fibrin at a desired time from the circulation of the rat. In these studies we modified the technique developed by Spar and co-workers by careful *in vitro* titration of the goat-anti-rabbit IGg, in terms of mg. of protein. Then rats bearing kidney tumor surgically implanted were IV injected with an *in vivo* screened ^{125}I-labeled normal rabbit IGg supplemented by rabbit serum containing the corresponding amount of γ-globulin to be expected in the rat circulating blood. Twenty-four hours after injection, a second IV injection of goat antibody to rabbit γ-globulin containing the predetermined units of goat antibody needed to procipitate all rabbit IGg circulating in the rat blood was given. Rats were killed by guillotine 30 minutes after the second injection. Our results indicate that the second antibody lowers the blood values by a factor of 10. Values for kidney tumor were 2-3 higher than blood, and for abdominal metestases, the ratios varied from 3-10. For normal kidney the ratio was close to blood values. For mesentery enlarged lymph nodes the ratio was increased by a factor of 6. Lungs, spleen and liver values were higher by a factor of 1, 12, and 10 respectively. These studies were compared with rats sacrificed by perfusion instead of guillotine. These results showed very little difference except for liver, kidney and lungs, ratios were lower.

This immunological technique was also carried out in F-344 rats bearing surgical tumor implants in the liver. In these experiments all rats were perfused. Results were similar to the values found for kidney tumor, except that in the case of liver implanted tumors the number of metastases found was increased.

Although the results of these studies are difficult to summarize, if one considers that these studies were done in an attempt to reproduce a human metastasizing tumor in the rat, our results seem to indicate that the extra-vascular-extracellular compartment in tumors, whether the tumor is implanted subcutaneously or in abdominal organs, does sequester γ-globulin in its extravascular-extracellular compartment, and that this process is a slow one.

Significance.

Our results indicate that our preparations of organ-specific antibody do localize to some degree, in mammary gland and mammary tumors, but the degree of localization may depend on the burden of circulating antigens in the blood and also in the rate of exchange into the extravascular-entracellular space of tumors. This possibility was confirmed by the immunological technique in which liver and spleen values were comparable to the high values obtained with organ specific antibody while tumor values were slightly higher for our preparations of organ-specific antibodies. Also our preparations of labeled antibody bind strongly to tumors. This was shown by the ^{125}I found in tumors 5 to 6 days after intravenous injection of labeled antibody.

Some possibilities for the immunological technique. One of the most common diagnostic tools for human cancer is CEA (carcino-embryonic antigen). It may be possible to obtain better photoscans if CEA circularing antigen in the blood stream of the patient is first removed by cold CEA antibody then 24 or 48 hours later the ^{131}I-labeled CEA goat antibody is administered. Reports in the literature indicate that ^{131}I-labeled CEA antibody is capable of showing up in scans even though the patient has high levels of circulating CEA antigen, and the amount of ^{131}I found in tumor is 0.001% of the injected dose. Our tumor and organ antibody preparations in terms of per cent dose per gram are closer and above 1.0.

[22] Iodine Monochloride (ICl) Iodination Techniques

By M. ANGELES CONTRERAS, WILLIAM F. BALE,* and IRVING L. SPAR

For labeling proteins and related substances in solution with ^{131}I or ^{125}I, the use of the ICl isotopic exchange procedure may often be the method of choice. The actual procedure is simple, since the reagents used are stable over long periods of time. In this technique the total amount of iodine incorporated into the iodinated material is known and can be controlled, and it avoids the use of oxidizing agents that may damage protein. These advantages were pointed out by McFarlane,[1] who developed an early version of this labeling procedure.

The basic method, as it is most widely used today,[2] is as follows: ^{131}I or ^{125}I in the form of iodide ions (often described by commercial sources as NaI) are mixed with the protein solution to be iodinated, buffered to a pH of approximately 8. An appropriate amount of ICl, in a weakly acidic NaCl solution, is then stirred rapidly with the protein–NaI mixture. The total buffering capacity must maintain the entire mixture at a pH of approximately 8.

As a result of this addition of ICl, two reactions occur. Radioactive iodine, e.g., ^{125}I, present as iodide, is converted to ^{125}ICl by very rapid chemical exchange with nonradioactive ICl. Iodination of protein takes place; it can be considered to occur as a result of the following reactions.

$$ICl + NaOH \rightarrow HOI = NaCl$$
$$HOI + \text{tyrosine residue} \rightarrow \text{iodinated tyrosine residue} + H_2O$$

This latter reaction is also rapid, so in 1 min a protective protein may be added and the labeled preparation be removed from the reaction vial.

Scope of Usefulness

Since the only oxidizing agent present during the iodination procedure is the ICl itself, no harm can come to protein that would be produced by other oxidizing agents. This contrasts with the oxidizing effects of chloramine-T used to oxidize iodide to iodine in the labeling procedure of that name. This advantage may sometimes be important when working with labile proteins. Since the amount of total iodine incorporated in the la-

* Deceased, June 28, 1982.

[1] A. S. McFarlane, *Nature (London)* **182,** 53 (1958).

[2] W. F. Bale, R. W. Helmkamp, T. P. Davis, M. J. Izzo, R. L. Goodland, M. A. Contreras, and I. L. Spar, *Proc. Soc. Exp. Biol. Med.* **122,** 407 (1966).

ISBN 0-12-181992-2

beled protein can never exceed the amount added as ICl, this sets an upper limit on the average number of iodine atoms attached to each molecule of labeled protein. This level of attached iodine is sometimes uncertain by other labeling procedures, since it cannot be readily controlled.

Three variants of the basic ion-exchange ICl iodination method will be considered. First to be described is a macro method in which the amount of protein to be labeled amounts to several milligrams, and the volume of solution in which it is contained is in the range of a few milliliters. This procedure is easily adapted to remote handling techniques behind γ-ray shields and has been used for attaching to 4 mg of immunoglobulin (IgG) 100 mCi or more of ^{131}I. Second to be described is a micro method for labeling amounts of protein ranging from less than 100 μg to several milligrams. The volume of the product after completing the labeling operation can be held to less than 1 ml. It is a good general procedure, but has not yet been adopted for remote shielded handling of large amounts of radioactivity. The third procedure to be described is a variant of the second in which the labeling procedure is carried out directly in suitable ^{125}I or ^{131}I shipping vials. Finally there will be a short discussion of techniques in which radioactive ICl is produced by oxidation of total iodide in radioactive NaI preparations.

Investigators proposing to use the ICl method for labeling should be aware of two limitations of this method. Since in this procedure no oxidation of iodide ion is carried out in the presence of protein to be labeled, any reducing substances present can compete for positive iodine with this protein. Therefore the presence of such substances should be kept low. Also, the ICl method has not been found to be a useful procedure for directly labeling cells or cell membranes; it is useful in preparing reagents for other types of ^{125}I or ^{131}I labeling—e.g., the preparation of diazotized diodosulfonilic acid for labeling of cell membranes.[3]

Reagents

Iodine Monochloride Reagent. A convenient stock solution of ICl is 0.02 *M* in ICl, 2.0 *M* in NaCl, 0.02 *M* in KCl, and 1.0 *M* in HCl. It should be noted that iodine monochloride has a long history as a convenient term for referring to what is more strictly the hydrochloric acid solution of the trihalide ion (ICl_2^-). As described more fully elsewhere,[2] it can be prepared as follows: To a solution of 0.5550 g of KI, 0.3567 g of KIO_3, and 29.23 g of NaCl is added 21 ml of concentrated HCl (sp. gr. 1.18) and water to make the volume to 250 ml. The slight amount of free iodine is

[3] R. W. Helmkamp and D. A. Sears, *Int. J. Appl. Radiat. Isot.* **21**, 683 (1970).

then removed by passing through the solution a current of air saturated with water vapor for a few hours. It is worthwhile to ascertain that the air for aeration is truly saturated and that no reduction in volume occurs during this aeration procedure. Completeness of free iodine removal is tested by extracting a few milliliters of the ICl preparation with CCl_4. The separated CCl_4 fraction should be colorless with only a faint pink blush when the extraction is complete. If care has been taken that the air being used for iodine extraction is fully water saturated, the final ICl solution should be within 1% of the calculated 0.02 *M* value. It is indefinitely stable at room temperature. The exact molarity of the solution can be determined by adding an excess of KI to an aliquot and titrating the liberated iodine with a standardized thiosulfate solution.

^{125}I Solutions. Several sources of ^{125}I-labeled iodide solutions are available suitable for use by procedures described here. High specific activity preparations prepared without reducing agent are preferable. For use with the micro method, some necessary restrictions on volume and alkali content will be given later.

Some quantitative knowledge of the characteristics of ^{125}I are needed in setting up procedures that give a satisfactory degree of ^{125}I coupling to protein and little or no damage to the labeled protein by attaching too much iodine to it. Pure ^{125}I contains 4.596×10^{-7} mole or 57.44×10^{-6} g of iodine per Curie, which by definition is undergoing 3.7×10^{10} disintegrations per second. Let it be assumed that 10 mCi of ^{125}I, 4.596×10^{-9} mol, without additional iodine present as impurity, is used to label 4 mg of IgG, and that 4 mol of ICl are used per mole of protein (that is, 4 molecules of ICl per protein molecule). Four milligrams of this protein equals 2.5×10^{-8} mol, and the ICl to be used is $4 \times 2.5 \times 10^{-8} = 1.0 \times 10^{-7}$ mol. Thus after complete isotopic equilibrium has been reached in the exchange reaction between iodide and ICl, the fraction of ^{125}I available for labeling will be $(1.0 \times 10^{-7})/(1.0 \times 10^{-7} + 4.596 \times 10^{-9}) = 0.9541 = 95.6\%$.

Even when the amount of IgG to be labeled is reduced to 1 mg, and the amount of ICl is reduced accordingly, still most of the ^{125}I, in this case 84.5% is available for labeling. The situation changes significantly when 0.1 mg of IgG is to be labeled with 10 mCi ^{125}I, at the previously considered ratio of 4 mol of ICl to 1 mol of IgG (1 ICl molecule per 40,000 daltons protein). The protein represents 6.25×10^{-10} mol of IgG, and the corresponding ICl equals 2.5×10^{-9} mol. Thus, after the exchange reaction is completed, the fraction of ^{125}I available for labeling will be $(2.5 \times 10^{-9})/(4.596 \times 10^{-9} + 2.5 \times 10^{-9}) = 0.352$, or 35.2%.

The fraction of ^{125}I available for iodination can be increased by using larger amounts of ICl, but at the same time increasing the possibility of protein damage by overiodination. However, one may note that the label-

ing of hormones with ^{125}I by the chloramine-T method for radioimmunoassay is often carried out at levels of 50–100 mCi of ^{125}I per milligram of protein.[4]

^{131}I Solutions. Stable ^{127}I and ^{129}I (1.6×10^{-7} year half-life) are produced in substantial amounts.[2,5] when ^{131}I is produced by neutron activation of normal tellurium, probably the method most used at the present time. If the neutron radiation time is 24 hr, and the resulting iodine is processed and shipped during the next 48 hr, the iodine at shipping time would be 44% ^{127}I, 23% ^{129}I, and 33% ^{131}I. Thus, in practice, it is difficult to obtain and use in the laboratory ^{131}I in which the ^{131}I exceeds 20% of the total iodine content.

Increases in the time of irradiation and delays in processing, shipping, and use can lead to large increases in non-^{131}I iodine. For example, if the neutron irradiation time is 10 days, and the time before use for iodination another 8 days, ^{131}I will amount to only 12% of the total iodine. Occasionally iodine obtained from suppliers contains substantial amounts of nonradioactive iodine which have been added to minimize absorption effects. Such iodine is entirely unsuited for labeling purposes, and the labeling efficiencies achieved are close to zero.

Iodine-131 less diluted by other iodine isotopes can be obtained as iodine produced by uranium fission. Such iodine has been used in experimental and clinical cancer therapy attempts with antibody carrying doses of ^{131}I in the therapeutic range.[6,7] Tellurium, highly enriched in ^{130}Te, which was produced at Oak Ridge National Laboratory, would be an ideal source for ^{131}I production. By this latter procedure, iodine could be produced that would be almost entirely ^{131}I.

Borate Buffer. This buffer, pH 8, is prepared by adjusting a distilled water solution of 0.16 *M* NaCl and 0.20 *M* H_3BO_3 with 1.6 *M* NaOH to pH 8, with a final NaOH concentration of approximately 0.04 *M*. Borate buffer with twice these amounts of NaCl and H_3BO_3, adjusted to pH 7.65 with 1.6 *M* NaOH, is termed 2× borate buffer. When diluted with an equal volume of distilled H_2O, the pH becomes 8.

Protein Solutions for Labeling. The protein to be labeled is dissolved or dialyzed against borate buffer. The glycine buffer suggested by McFarlane.[1] is also satisfactory but gives slightly lower labeling yields. As

[4] D. N. Orth, this series, Vol. 37, p. 22.

[5] W. F. Bale, R. W. Helmkamp, T. P. Davis, M. J. Izzo, R. L. Goodland, and I. L. Spar, "High Specific Activity Labeling of Proteins with ^{131}I," Report UR-604, AEC TID-4500 (16th ed.). 1962.

[6] I. L. Spar, W. F. Bale, D. Marrack, W. C. Dewey, R. J. McCardle, and P. V. Harper, *Cancer* 20, 865 (1967).

[7] W. C. Dewey, W. F. Bale, R. G. Rose, and D. Marrack, *Acta Unio Int. Cancrum* 19, 185 (1963).

noted earlier, any reducing substances present in the iodination mixture can compete with tyrosine residues for positive iodine. One common source of such reducing substances is cellophane dialysis tubing, particularly if the substance to be labeled has been concentrated in such tubing under reduced pressure. Therefore, if dialysis tubing is used, it should be purchased or extracted so as to be as free of reducing substances as possible. Proteins concentrated by reduced-pressure dialysis in cellophane or other membranes containing reducing substances that may be solubilized, should be dialyzed further to remove such reducing substances.

Under some conditions to be mentioned later, dialysis against 2× borate buffer may be preferable because of its greater buffering power.

Gel Separation Columns. For separating bound and unbound radioactive iodine, Sephadex G-25 or G-50 may be used. The size of the column should be such that an adequate separation is achieved between the fractions containing the labeled protein and the unreacted iodine or iodate. It is best to first ascertain the void volume with Blue Dextran and the point of inorganic iodine breakthrough using a small test sample of unreacted radioactive iodide. When passing the final labeled preparation through the column, the first burst of radioactivity should appear just behind the void volume for proteins in the immunoglobulin size range.

Ion Exchange Column. Dowex 1-X4 resin (50–100 mesh) is allowed to stand in contact with 1 *N* HCl for several hours, then washed successively with 20% NaCl solution, 0.85% NaCl solution until the filtrate is neutral, and then stored under the latter solution. A column is formed by placing 2.5 ml of this resin in a 3-ml disposable syringe containing a small plug of glass wool. The syringe is filled with 0.85% NaCl and then drained just before use.

It was found that such a column removed all iodide ions from an iodinated protein solution, and that no significant iodide ion was eluted on subsequent washing of the column with 1.5 ml of normal rabbit serum or 0.5 ml of this serum followed by 1 ml of saline solution. Despite this rinse, however, 4–8% of the iodinated IgG was still retained by the resin.

Catalase Solution. Sterile preparations, without preservatives, assaying 30,000 units or more per milligram of protein are used to destroy H_2O_2 that is sometimes present in high-activity commercial ^{131}I preparations.[2]

Macro Method for ^{125}I or ^{131}I Labeling

Apparatus

The setup shown in Fig. 1 is widely used for low and high level labeling with ^{131}I and ^{125}I and is well adapted for remote handling and good radia-

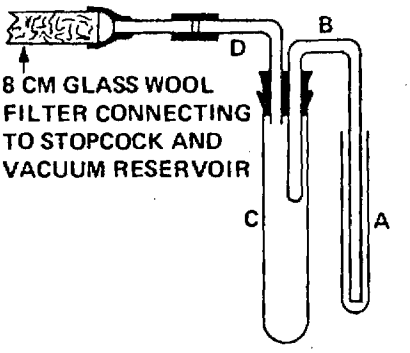

FIG. 1. Apparatus for iodination by the macro technique. Test tubes used at location A can be of any appropriate size to hold reagents. C is a 2.2 cm internal diameter × 15 cm Pyrex test tube. B is a 0.1 cm internal diameter capillary tube, slightly constricted at the end in tube C. Its effective aperture is such that the application of strong suction through D will jet 5 ml of water from A to C in about 1 sec.

tion shielding for ^{131}I preparations in the 100–300 mCi range. A combination of lead bricks and transparent lead glass shielding, of thick-walled lead tube holders, and remote-handling grip tools that can work around corners make isotopic labeling followed by ion exchange or molecular sieve passage possible with negligible exposure to the operator.

This procedure will first be described for labeling 4 mg of IgG, assumed molecular weight 160,000, dissolved in or dialyzed against borate buffer, with 4 equivalents of ICl. This is a level of 1 ICl molecule per 40,000-dalton protein.

The necessary dilution of ICl from the 0.02 *M* stock solution is calculated, and a subdilution is made as follows: 4 mg of IgG represents 2.5×10^{-8} mol of protein; four equivalents of ICl is therefore 1×10^{-7} mol. To provide this amount of ICl in 0.2 ml of solution, a dilution containing 5×10^{-7} mol/ml is needed. One milliliter of the stock ICl solution will contain $0.02/1000 = 2 \times 10^{-5}$ mol. Therefore the dilution factor needed will be $(2 \times 10^{-5})/(5 \times 10^{-7}) = 40$.

Step 1. To make this dilution 0.2 ml of the stock ICl solution is mixed with 7.8 ml of 2 *M* NaCl solution already added to a clean disposable test tube. (In a labeling procedure where a different amount of ICl is required, the dilution is calculated and made in such a way that the required amount of ICl is present in 0.2 ml of solution close to 2 *M* in NaCl.)

Step 2. The protein to be iodinated, in a volume of 3 ml or less, is placed in tube C, and the iodination apparatus is assembled. Alternatively, it may be added by suction through tube B after the apparatus is assembled.

Step 3. The radioactive NaI solution, made to a volume of 2–3 ml with borate buffer, is added to the protein solution by suction.

Step 4. Immediately 0.2 ml of the subdilution of ICl prepared under step 1 is added to 1.8 ml of 0.85% NaCl solution and mixed. Then, by rapid suction, this preparation is added as a jet to the iodine–protein solution.

Step 5. Labeling occurs rapidly, so 1 min later, 1 ml of protective protein (20–60 mg) is added to protect against radiation effects and destruction of protein that may occur by such phenomena as surface denaturation in dilute solution. Typical protective proteins used are 6.25% solutions of human or animal albumin, citrated human or animal plasma, normal serum, or a solution of gelatin.

Unbound radioactive iodine can be removed from the labeled preparation by ion-exchange resin, dialysis, or passage through a suitable gel filtration medium, such as Sephadex G-25.

The ion-exchange resin is probably the easiest to use for moderate to high molecular weight proteins such as IgG. After passage of the labeled preparation through the resin at the rate of about 1 ml/min, the resin is rinsed with an additional 1–1.5 ml of protective protein. This procedure has a disadvantage in that the resin does not retain iodate, and with a rinse of 1 ml of normal serum it still retains a few percent of IgG. However, the presence of iodate is rarely, if ever, a practical problem, except in old ^{125}I-labeled preparations. Also, it can be tested for by a preliminary passage of a small portion of the radioactive preparation to be used for iodination through the resin. Iodide will be retained by the column, but iodate will come through in the first 10 ml of the effluent. If the iodinated material is one retained by the resin, such as insulin, glucagon, and many other substances of relatively low molecular weight, dialysis or gel filtration can be used. Dialysis has the disadvantage that dialyzable radioactive iodine is distributed through large volumes of fluid that may present a disposal problem.

Radioisotope calibrators, of the type used in nuclear medicine, are convenient for measuring the ^{131}I content of different fractions during iodination procedures, but almost any equipment capable of giving good relative γ-ray intensity measurements can be utilized.

This general procedure has proved to be well adapted for the attachment of ^{131}I to antibody for the purposes of human cancer therapy. Here it is desirable that relatively large amounts of ^{131}I be attached to protein, 100 mCi or more for individual patients, so that the amount of administered foreign protein can be kept small, not more than 4–5 mg if possible if the antibody is a protein of xenogenic or allogenic origin, to slow or avoid the possibility of allergic sensitization. Up to the time iodination is complete,

the ^{131}I is transferred only by suction into closed containers, greatly reducing the possibility of radioactive aerosol formation. Equipment can easily be devised for carrying out the necessary procedures in a hood behind shields of lead or lead glass.

Use of Catalase

In earlier versions of the ICl iodination procedure,[8] in which radioactive ICl was formed first and then jetted into the substance to be labeled, it was found that hydrogen peroxide generated by the action of ^{131}I β-rays on water, first oxidized any reducing substances present and then produced free H_2O_2 that was able to destroy ICl. This reduced or prevented iodination. This H_2O_2 effect is reduced by the procedure in which ICl is added to the protein mixture, as described here, but this effect has not been studied at high ^{131}I levels. Catalase under these circumstances may still be useful in increasing ^{131}I iodination efficiency. As noted earlier, the catalase preparation should be of such purity that the amount used should be, in total protein content, a very small fraction of the material to be iodinated with ^{131}I.

To use catalase for H_2O_2 destruction before the labeling procedure is carried out, the $Na^{131}I$ solution should first be adjusted to approximately pH 8; 600 units of catalase are added, the mixture is allowed to stand for 10 min, and then step 3, the addition of the $Na^{131}I$ to protein, is carried out. Protein, added as catalase, is 0.5% or less of the 4 mg of protein to be coupled to ^{131}I.

Micro Method for Radioactive Iodine Labeling

The apparatus shown in Fig. 2 can in principle, and often in fact, be used for labeling protein in approximately the same amounts as in the macro method just described. However, procedures have not been developed for using it in a completely shielded operation. It has the advantage that, since the volumes used are small compared to those used in the macro method, protein concentrations can be kept higher during equivalent labeling procedures, and the effects of surface denaturation can be reduced.

The general procedure adopted to labeling 0.1-mg portions of IgG with 1-mCi amounts of ^{125}I, and some of the precautions to be taken, is as follows:

Step 1. A 1-inch, 21-gauge needle is inserted partially (0.5 inch) through the vial septum, and a 3.0-ml disposable syringe barrel, packed

[8] R. W. Helmkamp, R. L. Goodland, W. F. Bale, I. L. Spar, and L. E. Mutschler, *Cancer Res.* 20, 1495 (1960).

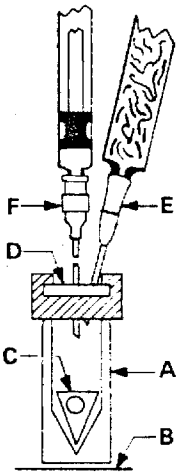

FIG. 2. Apparatus for iodination by the micro technique. A is the iodination V-vial.* B is the magnetic stirrer top. C is a triangular matrex Teflon-coated magnetic stirrer bar.* D is a self-sealing laminated disk.* E is a 21 gauge, 1-inch needle attached to a 3-ml disposable syringe barrel that has been packed, not too firmly, with glass wool. F represents a series of three hypodermic needles and syringes used in sequence during the iodination procedure. Stainless steel needles are recommended because of their greater strength. Lock-type syringes and stainless steel needles should be used when possible. Two-inch, 18 gauge needles are required for removing the labeled material from the iodination vial and often are necessary in transferring radioactive iodine from the shipping container to the iodination vial.
* Purchased from Pierce Chemical Co., Rockford, Illinois.

with glass wool, is attached to the needle. (This step may be omitted if great care is taken that subsequent steps are conducted in such a way that positive pressure does not build up inside the vial).

Step 2. Preparation of Diluted ICl. The amount of protein to be labeled by this procedure, 0.1 mg, is 1/40th (2.5%) of the amount used with the macro procedure just described. Therefore at the same protein: ICl ratio used for the macro procedure, an additional 1:40 dilution will be required. This is obtained by making the first 1:40 dilution described under the macro procedure. It will be used for making a further 1:40 dilution by mixing 0.2 ml of this preparation with 0.85% NaCl solution as a part of step 5.

Step 3. The protein to be labeled, preferably not exceeding 0.2 ml in volume, is added with a tuberculin syringe that is equipped with a 20-gauge needle and has been prerinsed with borate buffer.

Step 4. The $Na^{125}I$ solution, preferably not exceeding 0.2–0.3 ml in volume, diluted if necessary in borate buffer, is added with a syringe attached to an 18-gauge needle prerinsed with buffer. If feasible, it is

desirable that the needle be locked to the syringe, since accidental disengagement at this union has been the site of at least one iodine spill.

Step 5. Iodination. The rotating vane is actuated briefly to mix protein and ^{125}I solutions. Then the final dilution of ICl is made by mixing 0.2 ml of the first 1 : 40 dilution to a tube containing 7.8 ml of 0.85% NaCl solution. Then 0.2 ml of this 1 : 1600 dilution of the stock ICl solution is drawn into a tuberculin syringe and, with the vane rotating briskly, expelled rapidly through the septum into the $Na^{125}I$–protein mixture. The vane is stopped after 2–3 sec.

Step 6. One minute later, protective protein is added and the labeled protein is withdrawn with a syringe with a lock-type attachment to a 2-inch, 18-gauge needle. The labeled protein is passed through the resin column or another equivalent method for removing unbound radioactive iodine. The reactive vial is then rinsed with an additional amount of protective protein that is passed through the inorganic iodide removal apparatus.

Labeling in Shipping Containers

Some shipping containers, for example, the Combi-V-Vials supplied by New England Nuclear, can be used directly as reaction vessels for iodination. The main requirements are (*a*) a self-sealing septum through which reactants can be added and removed; (*b*) a vial volume adequate to accommodate the necessary reagents and mixing procedures; and (*c*) a method for rapid mixing during the addition of ICl. Experience shows that a Genie-Vortex mixer can be used with the Combi-V-Vial to provide such mixing. However, it is desirable to carry out preliminary tests with a nonradioactive vial and visual control to assure that mixing is adequate with the volumes of reactants used during the iodination procedure.

This third method, a modification of the micro procedure for labeling 0.1 mg of protein with 10 mCi of ^{125}I, will not be described. ^{125}I should be shipped at high concentration (>350 mCi/ml) in 0.1 *N* NaOH. Protein to be labeled should be dissolved in or dialyzed against borate buffer at as high a concentration of protein as feasible and then diluted in borate buffer (made from water and reagents free of reducing substances) to a level of 0.5 mg of protein per milliliter.

Step 1. A 1 : 40 dilution of stock 0.02 *M* ICl is prepared as described under step 2 of the micro iodination procedure.

Step 2. Protein to be labeled, 0.1 mg in a volume of 0.2 ml, is injected with a syringe into the vial containing $Na^{125}I$, and the two components are mixed. This may be done by inversion of the vial after the protein addition, to wash down any ^{125}I adhering to top of vial, or by a preliminary centrifugation before adding the protein.

Step 3. Iodination. This step is the same, in principal, as the step 5 of the micro iodination procedure. The final dilution of ICl is made by mixing 0.2 ml of the 1 : 40 dilution with 7.8 ml of 0.85% saline. Then, while the ^{125}I-protein mixture in the shipping vial is swirled with the Vortex mixer, 0.2 ml of the final ICl dilution (1 : 1600) is added rapidly by syringe. The mixing is stopped in 3–4 sec.

Step 4. One minute later, the protective protein is added. The continuing procedure is the same as described under step 6 of the micro procedure.

Iodination Using Radioactive ICl Produced by Oxidation of Total Iodine in NaI Preparations

Helmkamp *et al.*[9] have described a method for converting ^{131}I of Na^{131}I preparations quantitatively to ICl. No exchange reaction is involved. The original paper should be consulted for details. It has the advantage that all the radioactive iodine can be utilized for iodination, no matter how much there may be. This technique has the corresponding disadvantage that the actual amount of total iodine incorporated in the labeled material will be unknown unless one knows in advance the total iodine content of the Na^{131}I preparation used. Helmkamp and co-workers[10] have also developed a technique for determining the total iodine content of such Na^{131}I preparations.

In this labeling procedure initially designed for labeling 4 mg of γ-globulin with 50–200 mCi of ^{131}I, any H_2O_2 present in the Na^{131}I preparation is destroyed by catalase. The solution is then made acidic (pH approximately 3) with 0.25 *N* NCl. Saturated NaCl solution and 1 *N* NCl are then added in amounts such that, on subsequent addition of KIO_3 containing 5 times the estimated total iodide content of the Na^{131}I preparation used for labeling, the solution will be approximately 0.05 *N* in NCl and 1 *M* in NaCl. Under these conditions the iodide ion is converted to ICl with reasonable rapidity; the iodate ion undergoes a negligible amount of ion exchange. Thus at the end of 10 min that mixture can be adjusted rapidly by addition of a calculated amount of NaOH to a pH around neutrality and jetted into the solution to be iodinated buffered to a pH of 8. From then on, the procedure follows the ICl exchange procedure described above.

Doran and Spar[11] have described an adaptation of the oxidative ICl iodination technique adapted to labeling microgram amounts of protein

[9] R. W. Helmkamp, M. A. Contreras, and M. J. Izzo, *Int. J. Appl. Radiat. Isot.* **13**, 747 (1967).

[10] R. W. Helmkamp, M. A. Contreras, and W. F. Bale, *J. Nucl. Med.* **7**, 491 (1966).

[11] D. M. Doran and I. L. Spar, *J. Immunol. Methods* **39**, 155 (1980).

with ^{125}I to high levels of specific radioactivity. They include a review of the qualities of the iodinated proteins produced by the ICl and other iodination methods.

Additional Considerations

Although only two examples of labeling of proteins with radioactive iodine are discussed in detail, it is generally simple to extrapolate from them to suitable procedures for different amounts of protein to be labeled, different levels of radioactivity, alternative iodine isotopes, and different ratios of moles of iodine to moles of protein.

In general, ^{125}I can be substituted for ^{131}I, and vice versa, with no change in procedure. The amount of radioactive isotope used can vary from a small fraction of 1 μCi to many millicuries provided volumes are kept to the described range and proper safety precautions are taken. The molar ratio of ICl to protein can be varied, except that one must take into account the fact that decreasing the molar ratio of ICl to protein increases the effect of reducing substances in a protein solution, and the fraction of radioactive iodine incorporated into protein may be reduced.

It is important to calculate and carry out ICl dilutions so that a desirable molar ratio of ICl to protein is used. It is common and reasonable to assume, with larger proteins, that one ICl per 40,000 daltons protein is a reasonable level. The examples given here, labeling at a level of 4 molecules of ICl per IgG molecule of assumed 160,000 molecular weight, represents this level. There is, however, reported evidence that some proteins, e.g., fibrinogen, are damaged at this level of labeling. When labeling proteins with a molecular weight less than 40,000, a molecular ratio of 1 between protein and ICl represents a reasonable starting point. A final criterion is that the labeled protein fulfills the function for which the labeling operation was carried out.

Probably the macro method of labeling should not be routinely used for labeling less than 2 mg of protein. Low iodination yields may result, since reducing substances are not easy to eliminate from reagents, and in addition, surface denaturation may produce unacceptable protein damage. On the other hand, the micro method has become the routine procedure in our laboratory for labeling 4-mg amounts of protein with 10 mCi of ^{125}I or ^{131}I.

It is necessary that the buffering capacity of the protein solution, or of the buffer added with it, be sufficient to bring the pH of the ^{125}I or ^{131}I solution to approximately 8, possibly from a much more basic pH, and then keep the reaction mixture at approximately pH 8 after addition of the acidic ICl solution. The procedures as described do not cover all possibili-

ties. As one extreme example, one investigator desired to iodinate, if possible, all tyrosine residues of a protein and used enough ICl to do so. This introduced so much acid with the ICl, that the yellow ICl color persisted and no iodination occurred. The remedy was to prepare and use an ICl of much higher molarity. Some investigators have attempted to use ^{131}I solutions that contained so much base that the pH remained very high after adding the protein solution, and the protein was denatured and precipitated.

Effect of Reducing Substances

Use of ICl iodination techniques, with a ratio of 1 ICl molecule per 40,000 daltons protein, never resulted in radioactive iodine attachment to proteins approaching 100%. There are probably multiple reasons for this, but the major one is reported to be the presence of reducing substances present as a part of the protein to be labeled or in the fluid where the reaction occurs. McFarlane[1] has suggested a preliminary oxidation of protein by iodine at pH 4.5 as a means of eliminating reducing properties of protein to be iodinated, but it has never been adopted as a working procedure. As noted earlier, one common source of reducing substances is dialysis tubing. In one series of experiments on the micro iodination technique, the use, as a diluent, of borate buffer that had been in contact with cellulose dialyzing tubing under various conditions reduced efficiency of ^{125}I coupling from values of about 50% to values ranging from 39 to 2.5%.

Importance of Rapid ICl Mixing after Addition

If complete mixing has not occurred during the ICl addition to the radioactive iodine–protein mixture before the ICl has reacted with protein or has otherwise been destroyed, then some radioactive iodine will remain as unexchanged iodide and have no chance to react with protein. This will result in lower labeling effectiveness. In some experiments it can also lead to misinterpretation of results unless taken into account. For example, let it be supposed that the labeled preparation was an enzyme, and that the experiment was intended to determine whether labeling with approximately one atom of iodine per enzyme molecule would lead to complete loss of enzymic activity. If mixing were so incomplete that the ICl reacted with only one-half of the enzyme molecules, then 50% would still be unlabeled and would retain full enzyme activity, irrespective of whether one iodine atom per enzyme molecule could destroy enzyme activity, or not.

Radiation Damage

Proteins are particularly susceptible to radiation damage when in dilute solutions, since the ratio of free radicals and peroxidies produced from irradiation of water to protein molecules is high. At a level of 10 mCi/ml the self-irradiation from ^{131}I is at a level of about 4,000 rad/hr. In one study,[12] ^{131}I-labeled rabbit antibody to rat fibrin was purified and irradiated with ^{60}Co γ-radiation or lightly filtered 250 KV X-radiation. In dilute solutions (less than 0.1 mg of protein per milliliter), 10,000 rad of ^{60}Co radiation rendered the uptake of the purified labeled antibody by rat fibrin to only 17% and at 40,000 rad to about 47%. Dosage of 200,000 rad of ^{60}Co γ-rays or 100,000 rad of X-ray reduced the specific uptake to virtually zero. At this latter dosage the presence of normal rabbit serum protein at a level of 8 mg per milliliter of irradiated solution reduced the radiation effects to approximately that produced by 10,000 rad.

It is important immediately to minimize radiation damage once the iodination reaction is complete. Such protective measures are the addition of nonspecific inert protein, dilution, then freezing, which are compatible with the intended use of the iodinated protein.

Efficiency of Coupling of Radioactive Iodine to Protein

Data from our own and other laboratories indicate that the macro ICl procedure, when used to couple radioactive iodine to protein at protein levels of a few milligrams and ICl levels of one ICl molecule per 40,000 daltons protein gives labeling levels of 50% or higher in most instances, usually at the 60–70% level. The major exceptions are instances where amounts of ^{131}I in the 100 mCi or higher levels were used, and the total amount of iodine involved was great enough to render the ICl exchange reaction an inefficient one. An extensive use of one adaptation of the macro method for insulin labeling gave preparations with relatively low efficiency of ^{131}I or ^{125}I coupling, but with reproducible production of biologically active insulin metabolized in a normal physiological manner.[13]

The use of the micro method is now standard in our laboratory for 10 mCi or less of radioactive iodine, and amounts of protein ranging from 0.1 mg to 4.0 mg. Labeling efficiencies are similar to the macro method at the

[12] R. W. Helmkamp, R. L. Goodland, W. F. Bale, I. L. Spar, and L. E. Mutschler, "High Specific Activity Iodination of Gamma-Globulin with Iodine-131 Monochloride," Report UR-568. AEC TID-4500 (15th ed.), 1960.

[13] J. L. Izzo, A. M. Roncone, D. L. Helton, and M. J. Izzo, *Arch. Biochem. Biophys.* **198**, 97 (1979).

level of 4 mg of protein, and much better, around 49–60%, when low millicurie levels of ^{125}I are used to label 0.13 mg of protein.

Isotopic Purity of ^{131}I Produced by Fission and from Tellurium

One currently experimental, but potential future routine, use of ^{131}I is as a therapeutic agent attached to tumor-localizing antibodies, either allogeneic or xenogeneic in nature. To diminish the amount of antibody that must be used, and keep low the possibility of allergic reaction, it is desirable to use ^{131}I of as high a specific activity as possible. The most common current method for producing ^{131}I, the irradiation of tellurium with slow neutrons, unavoidably also produces stable ^{127}I and ^{129}I with a 1.6×10^7 year half-life. The highest specific activity ^{131}I currently available contains approximately 100 μg of total iodine per Curie, of which only 8 μg is ^{131}I.

Table I shows the composition of iodine obtained after neutron bombardment of natural tellurium as a function of duration of irradiation and time after activation. Table II shows similar data for iodine produced by slow neutron-induced fission of ^{235}U. Earlier[2] our laboratory was able to obtain from Oak Ridge National Laboratory fission-produced ^{131}I with specific activities as high as 24 μg of total iodine per Curie of ^{131}I.

Inspection of Table I shows that this high specific activity is scarcely possible with ^{131}I obtained from natural tellurium. With sufficient demand,

TABLE I
ISOTOPIC PURITY OF ^{131}I PRODUCED FROM TELLURIUM[a]

Activation time (days)	Time after activation (days)	Percentage of total iodine			Micrograms total iodine per Curie ^{131}I
		^{127}I	^{129}I	^{131}I	
1	2	44	23	33	25
10	2	61	20	19	42
20	2	66	20	14	59
40	2	70	22	8	96
1	8	51	26	23	36
10	8	67	21	12	67
20	8	69	22	9	93
40	8	72	23	5	115

[a] Calculated relative amounts of neutron-induced iodine isotopes present 2 and 8 days after activation of natural tellurium in iodine separated immediately after activation.

TABLE II
ISOTOPIC PURITY OF ^{131}I PRODUCED BY FISSION OF ^{235}U[a]

Activation time (days)	Time activation (days)	Percentage of total iodine			Micrograms total iodine per Curie ^{131}I
		^{127}I	^{129}I	^{131}I	
1	8	0.1	22	78	10
10	8	3.0	34	63	13
20	8	5.0	42	53	15
40	8	8.0	55	37	22
1	32	0.4	68	31	26
10	32	7.0	76	17	47
20	32	10.0	78	12	66
40	32	12.0	81	7	116

[a] Calculated relative amounts of fission product iodine isotopes present 8 and 32 days after bombardment of ^{235}U in iodine separated immediately after activation.

fission-produced ^{131}I might again become available, or the production of ^{131}I from tellurium highly enriched in ^{130}Te become economically feasible.

Acknowledgments

This work was supported by USPHS Grant RO1 CA25958 from the National Cancer Institute, NIH, and by the Medical Research Foundation, Inc., Atlanta, Georgia.

[23] Application of High-Performance Liquid Chromatography to Characterize Radiolabeled Peptides for Radioimmunoassay, Biosynthesis, and Microsequence Studies of Polypeptide Hormones

By N. G. SEIDAH and M. CHRÉTIEN

Background

The use of radiolabeled peptides and proteins as tracers in radioimmunoassay (RIA), receptor binding, biosynthesis, and microsequencing studies has gained wide acceptance owing to the ease of preparation, the high specific activities obtainable, and the sensitivity of detection. High-performance liquid chromatography (HPLC) has been used to purify and characterize radiolabeled polypeptides as an efficient and rapid alterna-

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